Comparison of Bio-degradation for Ridge Preservation Using Silk Fibroin-based Grafts and a Collagen Plug

Hyun Seok¹ · You-Young Jo² · HaeYong Kweon² · Seong-Gon Kim³ ·
Min-Keun Kim³ · Weon-Sik Chae⁴

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Abstract A material for ridge preservation should have dimensional stability to resist bio-degradation. This study was designed to compare bio-degradation of ridge preservation materials. Collagen plug was used as a positive control. Untreated, ethanol-treated, and 4-hexylresorcinol (4HR)-treated silk plugs were used for the experimental group. Each material underwent a scanning electron microscopic exam and a Fourier transform infrared (FT-IR) spectroscopic exam. Bio-degradation was evaluated by analyzing cylindrical bony defects in rabbit tibias. There were no prominent differences in microstructure among the silk plug groups. FT-IR exam demonstrated that the ethanol- and 4HR-treated silk plug groups had enhanced β -sheet structure. All silk plug groups exhibited significantly higher residual graft than the collagen plug group 4 weeks postoperative ($p < 0.05$). In conclusion, silk fibroin-based ridge preservation material was less bio-degradable than a collagen plug until at least 4 weeks after grafting.

Keywords Silk fibroin · 4-Hexylresorcinol · Ridge preservation · Bio-degradation · Plug

1 Introduction

The dimensional stability of alveolar bone is dependent on the presence of teeth. After tooth extraction, bone loss is unavoidable [1]. The loss of width between the buccal side and lingual side is generally greater than that of vertical height [2]. Many clinical variables can influence the degree

of bony resorption such as the thickness of the buccal bony plate [3] and the flap elevation [4].

Severe alveolar bone loss is a challenging issue for dental rehabilitation. Dental implants cannot be installed into the ideal position in the atrophic alveolar ridge [5]. Severely atrophic alveolar ridges cannot provide enough stability to denture [6]. Thus, reconstructive surgical procedures are required for the rehabilitation of atrophic alveolar ridges. Recently, ridge preservation procedures have been performed after extraction for prevention of progressive bone loss [7]. The ridge preservation procedure is a simple surgical process consisting of insertion of a graft into the extraction socket. As an additional procedure, a collagen membrane [8] or free gingival graft [9] may be used to cover the graft.

Many types of graft materials have been used for ridge preservation [7]. Autogenous bone can be considered but has the disadvantage of donor site morbidity [10]. Bovine bone or collagen is used for ridge preservation as a xenograft [10, 11], as are calcium-containing alloplastic materials such as hydroxyapatite [12]. Unfortunately, none of

✉ Seong-Gon Kim
kimsg@wnu.ac.kr

¹ Department of Oral and Maxillofacial Surgery, Chungbuk National University Hospital, Cheongju 28644, Republic of Korea

² Sericultural and Apicultural Materials Division, National Academy of Agricultural Science, Wanju-gun 55365, Republic of Korea

³ Department of Oral and Maxillofacial Surgery, College of Dentistry, Gangneung-Wonju National University, Jukheon gil 7, Gangneung, Gangwondo 25457, Republic of Korea

⁴ Daegu Center, Korea Basic Science Institute, Daegu 41566, Republic of Korea

these materials have been shown completely preserve the ridge without the loss of bone [2, 11]. Collagen is too rapidly degraded and used as cross-linked form [13]. A collagen plug alone is often used for ridge preservation but has not shown positive results [14]. Xenografts such as bovine bone-based materials are good at maintaining dimensions, and rarely bio-degrade [15]. As graft materials occupy the alveolar bone space, the ratio of contact between dental implant and newly formed bone is decreased accordingly [14].

Therefore, an ideal material for ridge preservation should have proper bio-degradation velocity. Silk fibroin (SF) is produced by *Bombyx mori* [16] and is known to have slow degradation in the body compared to collagen [17]. SF is also known to be an osteogenic material [18] and can be used as a scaffold for bone grafts [19]. SF-based sponge has been developed as a hemostatic material [20]. Considering that bleeding is a common complication after extraction [21], SF-based ridge preservation materials should have advantages compared to collagen based materials.

The objective of this study was comparison of the (1) micro-morphology, (2) FT-IR absorption spectrum, (3) bio-

degradation, and (4) tissue response among silk fibroin-based materials and collagen plug.

2 Materials and methods

2.1 Materials

Collagen plug for ridge preservation was purchased from a commercial producer (Ateloplug[®], Bioland, Cheongwon, Korea). Silk plug was composed of SF matrix and provided by Rural Development Administration (Jeonju, Korea). Untreated silk plug was treated with 10% ethanol for 1 h or with 3 wt% 4-hexylresorcinol (4HR) in 10% ethanol for 1 h. All materials were sterilized with ethylene oxide (EO) gas before usage.

2.2 Scanning electron microscopic (SEM) analysis

The morphology of graft materials was observed by SEM. The graft material after EO gas sterilization was used for the micro-structure morphological evaluation. SEM image taking was referred to KITECH (Korea Institute of

Fig. 1 Scanning electron microscopic findings. **A** Collagen plug was bundles of collagen fibers. Uncoiled collagen fibrils were also found. **B** In case of silk plug, sheet-like structures and globular proteins were

found. The end of protein mass was round and resembles water-drop. **C** Ethanol-treated silk plug. **D** 4-hexylresorcinol-treated silk plug (Bar = 100 μ m)

Fig. 2 Fourier transform infrared (FT-IR) spectroscopic findings. **A** Infrared absorption spectra of the silk materials showed characteristic amide absorptions at the regions of 1600–1700 (amide I), 1480–1580 (amide II), and 1230–1300 cm^{-1} (amide III). Amide A absorption appears at $\sim 3300 \text{ cm}^{-1}$ due to N–H stretching vibrations.

B The amide I peak consists of several sub-band IR absorptions. **C** The second derivative spectrum of the IR absorption presents clearly the peak positions of the sub-band components (4HR 4-hexylresorcinol, EtOH ethanol)

Industrial Technology, Gangneung, Korea). Subsequent procedure was done in KITECH. The images were captured by SEM (Hitachi, SU-70, Japan) at 5 keV.

2.3 Fourier transform infrared (FT-IR) spectroscopy

A Vertex 80 (Bruker Optics, Billerica, MA, USA) equipped with an attenuated total reflectance (ATR) accessory (MIRacle, PIKE technologies, Fitchburg, WI, USA) was used for the measurement of the FT-IR absorption spectrum. Subsequent procedures were performed by the Korea Basic Science Institute (Daegu, Korea). Spectra were recorded in the spectral range of 600–4000 cm^{-1} at a resolution of 2 cm^{-1} using a mercury cadmium telluride detector. Each spectrum was the result of the average of 128 repeated scans.

2.4 Animal experiments

A total of 26, 12-week-old New Zealand white rabbits with an average weight of 2.3 kg were used for this experiment. This experiment was approved by the Institutional Animal Care and Use Committee of the Gangneung-Wonju National University, Gangneung, Korea (GWN-2016-23).

General anesthesia was injected intramuscularly with a combination of Zoletil (Bayer Korea, Seoul, Korea) and Rompun (Bayer Korea). The leg area was shaved and disinfected with povidone-iodine. Local anesthetic was given before the incision. A longitudinal incision was made in the tibia. Blunt dissection was performed for reflecting muscle, and the periosteum of the tibia was exposed. Another incision was performed on the periosteum and the periosteum was reflected from the tibia, exposing the tibial

Fig. 3 The area of residual graft. The area of residual graft at 2 and 4 weeks after grafting was shown as ratio of residual graft compared to original graft for collagen plug (A), silk plug (S), ethanol-treated silk plug (E), and 4HR-treated silk plug group (4HR) (* $p < 0.05$)

bone. A trephine bur (diameter: 5.0 mm) was used under copious saline irrigation to create a bony defect. Two holes were prepared in each rabbit. The grafts were prepared to the same size (diameter: 5.0 mm, length: 5 mm). A graft was randomly placed into the bony defect, and each of the three types of silk plugs (i.e., untreated, ethanol-treated, and 4HR-treated) was placed into the bone defects. As a positive control, a collagen plug was put into the bony defect. As a negative control, the bony defect was left vacant without any graft. The skin was closed with 3–0 silk. After the surgery, the animals were given antibiotics and analgesics intramuscularly three times daily for 3 days. Each rabbit was individually caged and fed. At 2 and 4 weeks after surgery, 13 rabbits at each time point were euthanized.

2.5 Histological analysis

Before the tissue processing procedure, the bones underwent decalcification using 5% nitric acid for 5 days. The tibial bone was separated around the previously grafted area. The segments were embedded to show cross-sections of the tibia in paraffin blocks. The paraffin blocks were sliced and stained with hematoxylin and eosin stain to evaluate the residual amount of graft. The area of residual graft was calculated using the image analysis program in the examined area. The ratio of the residual graft was calculated as the percent ratio between the original graft size and the graft size of the observation.

2.6 Statistical analysis

An ANOVA test was carried out to compare the five groups. The Bonferroni's method of multiple comparisons was also used as a post hoc test. The significance level was $p < 0.05$.

3 Results

3.1 Morphology of collagen and silk plug under SEM

SEM images demonstrated that the micro-structure of collagen plug was different from that of the silk plug (Fig. 1). The collagen plug was composed of bundles of collagen fibers (Fig. 1A). Uncoiled collagen fibrils were also found. In case of silk plug, sheet-like structures and globular proteins were found (Fig. 1B). The end of the protein mass was round and resembled a droplet of water. Prominent morphological differences among silk plugs were not found (Fig. 1B–D).

3.2 Structural characterization of collagen and each silk plug

The infrared absorption spectra of the silk materials showed characteristic amide absorptions at the regions of 1600–1700 (amide I), 1480–1580 (amide II), and 1230–1300 cm^{-1} (amide III) (Fig. 2A). Amide A absorption appeared at $\sim 3300 \text{ cm}^{-1}$ due to N–H stretching vibrations. The amide I peak, which originates from C=O stretching vibrations, can be sensitively shifted in the range of 1600–1700 cm^{-1} depending on the unique protein secondary structure and involved hydrogen bonding pattern [22]. Therefore, fine structure analysis of the measured ATR-based IR absorption spectrum has obvious benefits for revealing the relative abundance estimates of protein secondary structures.

The amide I peak consists of several sub-band IR absorptions, as shown in Fig. 2B. The second derivative spectrum of the IR absorption clearly presents the peak positions of the sub-band components (Fig. 2C). The bare silk shows the majority of its absorption at $\sim 1647 \text{ cm}^{-1}$, corresponding to the random coil secondary structure, as well as the minor contributions of the α -helix, β -turn, and β -sheet structures. When the silk plug was treated with 4HR and ethanol, it is notable that β -sheet structure (1626 cm^{-1}) became the major component, with reduced abundance of the random coil structure. Compared to the ethanol treated silk plug, the 4HR treated silk plug shows negligible absorption for the random coil. In particular, the commercial Ateloplug (collagen plug) shows distinguished abundance of the α -helix structure (1660 cm^{-1}).

3.3 Residual graft in histological analysis

The area of residual grafts 2 weeks after grafting were 11.25 ± 1.85 , 37.07 ± 18.14 , 66.27 ± 17.58 , and $59.17 \pm 22.88\%$ in collagen, SF, ethanol-treated SF, and 4HR-

Fig. 4 Histological findings at 2 weeks after grafting (hematoxylin and eosin stain). **A** Unfilled control (original magnification $\times 5$). **B** Collagen plug (original magnification $\times 5$). **C** Silk plug (original magnification $\times 5$). **D** Ethanol-treated silk plug (original magnification $\times 5$). **E** 4HR-treated silk plug (original magnification $\times 5$). **F** High magnification view (original magnification $\times 200$) demonstrated that unfilled control showed reticular fatty bone marrow. **G** Residual collagen fibers were shown as mesh-like fibers in collagen plug group (original magnification $\times 200$). **H** High magnification view for untreated silk plug (original magnification $\times 200$). **I** High magnification view for ethanol-treated silk plug (original magnification $\times 200$). **J** Interestingly, there were few cellular components in 4HR incorporated silk plug (original magnification $\times 200$)

Fig. 4 continued

treated SF group, respectively (Fig. 3). The difference among groups was statistically significant ($p < 0.001$). As reported by the post hoc test, the collagen group had a significantly smaller residual graft compared to the ethanol-treated SF or, 4HR-treated SF groups ($p < 0.001$ and

$p = 0.001$, respectively). The unfilled control showed reticular fatty bone marrow (Fig. 4F). Residual collagen fibers were shown as mesh-like fibers in the collagen plug group, with the cells having hyperchromatic nuclei and little cytoplasm scattered into the collagen mesh (Fig. 4G).

The silk plug had globular silk fibroin proteins, and cells similar to those found in the collagen plug were also present (Fig. 4H, I). Interestingly, there were few cellular components in the 4HR incorporated silk plug (Fig. 4J).

The areas of residual graft at 4 weeks after grafting were 3.24 ± 1.99 , 40.72 ± 21.70 , 46.92 ± 3.44 , and $43.77 \pm 12.06\%$ in collagen, SF, ethanol-treated SF, and 4HR-treated SF group, respectively (Fig. 3). The differences among groups were statistically significant ($p < 0.001$). In the post hoc test, the collagen group had a significantly smaller residual graft compared to the untreated SF ($p = 0.001$), ethanol-treated SF ($p < 0.001$), and 4HR-treated SF groups ($p < 0.001$), respectively. There was no prominent difference between 2 and 4 weeks in the unfilled control group (Fig. 3). Mature fatty bone marrow was found in the unfilled control group (Fig. 5F). Residual collagen was uncommon, and phagocytosed by multinucleated giant cells in the collagen plug group (Fig. 5G). Unlike the collagen plug group, many silk proteins were still found in silk plug groups. Large foreign body cells were found in the untreated silk plug group (Fig. 5H). However, few giant cells were present in the ethanol-treated silk and 4HR-treated silk plug groups (Fig. 5I, J). Interestingly, the 4HR-treated group showed massive infiltration of red blood cells (Fig. 5J).

4 Discussion

The ridge preservation procedure is performed to preserve volume after tooth extraction. Therefore, bio-degradation of a material that is too rapid is not beneficial as a graft for ridge preservation [7, 11]. Collagen based materials have been widely used for ridge preservation [14]. In this study, three types of silk plug showed higher resistance to bio-degradation than a commercially available collagen plug (Fig. 3). Signs of acute or chronic inflammation were rarely found around silk plugs (Figs. 4, 5). Interestingly, ethanol treatment could increase the resistance to bio-degradation. However, the difference in the amount of residual graft was not statistically significant between the untreated silk plug and ethanol-treated silk plug groups ($p > 0.05$).

The dimensional change after tooth extraction has been reported to be 29–63% in the bucco-lingual width and 11–12% in the vertical width at 6 months [2]. Particularly, excessive loss of the bucco-lingual width occurs within the first week after extraction in the absence of a ridge preservation procedure [23]. Immediate implantation after tooth extraction may present progressive alveolar bone loss [24]. However, immediate installation of a dental implant cannot completely prevent progressive bone loss [25]. Alloplastic bone graft materials, such as hydroxyapatite,

tricalcium phosphate, and titanium granule, have been associated with delayed bone healing [23]. While collagen plug has been introduced as a ridge preservation material, it has not contributed to ridge preservation because of rapid degradation [14]. The area of residual grafts 2 weeks after grafting was $37.07 \pm 18.14\%$ in SF group (Fig. 3). That at 4 weeks after grafting was $40.72 \pm 21.70\%$ in the same group. There was no significant change of residual grafts in SF group between 2 and 4 weeks after grafting because silk fibroin is slowly degradable biomaterials [17]. Materials that degrade quickly may not prevent volume loss at the alveolar ridge [26].

According to FT-IR spectroscopic results, ethanol or 4HR-treated silk fibroin showed enhanced β -sheet structure with a reduction in the random coil structure (Fig. 2). In contrast, collagen plug had abundant α -helix structure. Ethanol treatment for silk fibroin is known to increase β -sheet structure [27]. Decreased β -sheet structure can destabilize the protein structure and may accelerate bio-degradation [28]. Furthermore, 4HR is known to increase expression of proteolytic enzymes in macrophages [29]. Therefore, the 4HR incorporated silk plug might show accelerated degradation compared with the untreated silk fibroin plug. In this study, the average value of residual graft area was lower in the 4HR treated silk plug group than in the ethanol treated silk plug group (Fig. 3). However, the 4HR treated silk plug group had higher residual graft area than the untreated silk plug group. 4HR mediated bio-degradation is a macrophage-mediated response and is activated during the remodeling phase [29]. As 4 weeks after operation is too early for wound remodeling, the 4HR incorporated silk plug would not show significant volume change until 4 weeks. 4HR incorporated silk sutures show significant bio-degradation 9 weeks after grafting [29]. 4HR incorporated silk-based bone grafts also show significant bio-degradation 8 weeks after grafting [30]. However, excessive amounts of 4HR in bovine bone may result in sudden graft degradation [31]. Therefore, an optimal concentration of 4HR would be important for programmed bio-degradation.

In this study, 4HR incorporated silk plug did not show a significant difference in bio-degradation compared to untreated silk plug. Considering that 4HR is a well-known anti-septic [32], 4HR incorporated silk plug should have anti-bacterial properties. Since many kinds of bacteria are indwelling in the oral cavity, the anti-bacterial properties of 4HR will be beneficial for the ridge preservation material. Infection associated with bacteria is an important reason of graft failure in the oral cavity [33]. The drawback of this study was a short observation period in the animal study. However, considering that the basic requirement for ridge preservation material is keeping dimensional stability until 4 weeks after extraction, the

Fig. 5 Histological findings at 4 weeks after grafting (hematoxylin and eosin stain). **A** Unfilled control (original magnification $\times 5$). **B** Collagen plug (original magnification $\times 5$). **C** Silk plug (original magnification $\times 5$). **D** Ethanol-treated silk plug (original magnification $\times 5$). **E** 4HR-treated silk plug (original magnification $\times 5$). **F** High magnification view (original magnification $\times 200$) demonstrated that unfilled control showed mature fatty bone marrow. **G** Residual collagen was few and they were phagocytosed by multinucleated giant cells in collagen plug group (original magnification $\times 200$). **H** Big sized foreign body giant cells were found in untreated silk plug group (original magnification $\times 200$). **I** Ethanol-treated silk plug showed few giant cells (original magnification $\times 200$). **J** Interestingly, 4HR-treated group showed massive infiltration of red blood cells with few giant cells (original magnification $\times 200$)

Fig. 5 continued

observation period met the primary purpose of the comparative study. The bone regeneration capacity of silk plugs should be further evaluated in studies with long-term observation.

In conclusion, silk fibroin-based ridge preservation material was less bio-degradable than a collagen plug until at least 4 weeks after grafting. Ethanol treatment for silk fibroin could increase the resistance to bio-degradation.

However, the difference in bio-degradation between ethanol-treated silk fibroin and untreated silk fibroin was not statistically significant. 4HR treatment for silk fibroin did not induce significant bio-degradation until 4 weeks after grafting compared to untreated silk fibroin.

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Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest.

Ethical statement This animal experiment was approved by the Institutional Animal Care and Use Committee of the Gangneung-Wonju National University (GWNU-2016-23).

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